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ORIGINAL ARTICLE

Spectroscopic investigations on the interactions of potent platinum(II) anticancer agents with bovine serum albumin

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Abstract The interactions of three platinum(II)-based anticancer complexes [(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$, [(5,6-dimethyl-1,10-phenanthroline)(1*R*,2*R*-diaminocyclohexane)platinum(II)]$^{2+}$, and [(5,6-dimethyl-1,10-phenanthroline)(1,2-diaminoethane)platinum(II)]$^{2+}$ (56MEEN) with BSA have been examined by circular dichroism (CD), fluorescence and ^{1}H pulsed gradient spin–echo (PGSE) diffusion NMR spectroscopy. The number of association constants and sites differed depending upon the spectroscopic method. This may be because each technique monitors different types of interaction/s and/or as a consequence of the different concentration ranges required for each technique. The titration of BSA with the achiral 56MEEN as monitored by CD indicates a reduction in the α-helical nature of the albumin, with the association constant calculated to be ~5×10^{6} M^{-1} for one site. Due to the chiral nature of the other two complexes, their association with albumin was not monitored using CD but was examined using fluorescence and PGSE diffusion NMR. Titration of BSA with any of the three metal complexes resulted in quenching of fluorescence, with the number of association sites calculated to be ~1.1, with an association constant of ~2×10^{5} M^{-1}. PGSE diffusion NMR provided insights into interactions occurring with the BSA in its entirety, rather than with individual regions. Metal complex binding sites were estimated (~10 equivalent) from the diffusion data, with the average association constant for all sites ~10^{2}–$10^{3}M^{-1}$. These experiments highlight the information that can be elucidated from complementary spectroscopic techniques and demonstrate the usefulness of PGSE diffusion NMR in monitoring multiple weak binding sites, which is of great importance in studying drug-biomolecule interactions.

Keywords Platinum · PGSE NMR · Intercalator · Albumin · Binding · Diffusion · Spectroscopy

Introduction

The recent synthesis of a large family of platinum(II)-based DNA intercalators [3, 11, 23, 49] has led to the investigation of structure–activity relationships for compounds containing bidentate amines and substituted 1,10-phenanthroline. The most active of these compounds, 56MESS [(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)]Cl_2, displays cytotoxicity up to 1,000 times greater than cisplatin in several cancer cell lines [20, 49]. Its enantiomer, 56MERR [(5,6-dimethyl-1,10-phenanthroline)(1*R*,2*R*-diaminocyclohexane)]Cl_2 and the achiral analogue 56MEEN [(5,6-dimethyl-1,10-phenanthroline)(1,2-diaminoethane)]Cl_2 (Fig. 1) are less cytotoxic than 56MESS [3, 20, 49]. While it is known that these compounds are subject to degradation by glutathione in vitro [20], little is known as to the behavior of these compounds in vivo and their interaction with larger physiologically relevant biomolecules such as albumin.

Albumin is the most abundant protein in plasma (~40 mg mL^{-1}, 0.6 mM) [21], and is responsible for the transport and distribution of a wide variety of endogenous and exogenous substances [22], including fatty acids,

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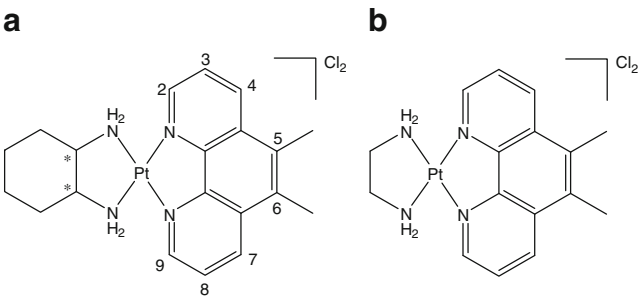

Fig. 1 The chemical structures of **a** 56MESS/56MERR and **b** 56MEEN. The numbering system for the 1,10-phenanthroline ligand is shown. * indicates a chiral center

hormones, metal ions and drugs. As such it is of interest to study the interaction/s of these platinum(II) intercalators with albumin.

Drug binding to albumin can influence the pharmacokinetics of the drug by affecting distribution and clearance [32]. The number of drug binding sites and their respective binding affinities affect the concentration of the free/active compound in the plasma. Strong binding to albumin can reduce the efficacy, distribution and clearance of the drug, and as such, weaker binding may be more useful for drug transport [7]. It has been suggested that over 90 % of intravenously administered cisplatin is covalently bound to plasma proteins such as albumin, transferrin, and γ-globulin [1, 14]. The major cisplatin binding site on albumin is believed to be Met-298 and, to a lesser extent, Cys-34 [19, 45]. It has been shown that up to 10 mol of platinum bind per mole of albumin, indicating the presence of multiple binding sites on the protein [44]. The association constant (K_a) for cisplatin to albumin is relatively weak (K_a=8.52×10^2 M^{-1}, as calculated by absorption spectroscopy) [30] in comparison to other drugs (10^5–10^6 M^{-1}) [22].

There is debate over the role of albumin in the antitumor effects and/or unwanted toxicity of cisplatin. Previously, it has been assumed that the irreversible covalent cisplatin–albumin adducts would be unable to deliver the drug to the target [5]; however, there are a number of reports where beneficial clinical effects are observed with administration of albumin–cisplatin adducts, compared to cisplatin alone [10, 16, 46]. In addition, patients with decreased plasma albumin levels experience increased toxicity when treated with cisplatin, combined with a poorer tumor response [9, 15]. While albumin plays a beneficial role in the drug delivery of some pharmaceuticals, its role in the transport of platinum-based pharmaceuticals is still uncertain.

There are a number of techniques available for probing the interactions of small molecules with proteins. One of the most frequently reported is X-ray crystallography; however, this technique does not represent the behavior in solution state. Circular dichroism (CD) spectroscopy measures the differential absorption of circularly polarized light by optically active chiral molecules. The predominantly α-helical secondary structure of bovine serum albumin (BSA; ~55 %) [30], allows CD spectroscopy to be utilized to monitor changes to the protein in these regions due to drug association. While BSA has a shorter amino acid sequence (583 compared to 585 residues) than human serum albumin, they share approximately 76 % sequence homology, with a similar three-dimensional structure and as such BSA is often used for spectroscopic studies [2, 39, 43].

Drug–albumin interactions can also be monitored by measuring the quenching of the inherent fluorescence of the tryptophan residue/s, providing information about structural changes of the protein and also the accessibility of the drug to the fluorophores. BSA is ideal for these experiments as it has two tryptophan (Trp) residues: Trp-134 is located on the surface of the molecule (domain I), and Trp-212 which is located within a hydrophobic pocket of the molecule (domain II) [22].

Pulsed gradient spin–echo (PGSE) nuclear magnetic resonance (NMR) is a non-invasive method for the measurement of self-diffusion coefficients in the solution state [4, 35]. The self-diffusion coefficient (D) of a molecule is related to its size and shape by the Stokes–Einstein–Sutherland equation [8, 42]:

$$D = \frac{kT}{6\pi\eta r} \quad (1)$$

where k is Boltzmann's constant, T is temperature, η is the solvent viscosity and r is the effective hydrodynamic radius of the molecule. Measuring the diffusion coefficient of various species in solution provides useful information about the interactions taking place between them, as the diffusion coefficient is a measure of the size and shape of a molecular species. PGSE NMR has been demonstrated as a technique for determination of drug–albumin binding constants [34].

The role of abundance, flexibility and binding affinity of albumin and the effects on the distribution of hydrophobic drugs in plasma are important factor to consider in the development of novel platinum chemotherapeutics. In this study, the interactions of albumin with 56MESS, 56MERR, and/or 56MEEN were investigated using CD spectroscopy, fluorescence spectroscopy and PGSE NMR to determine the strength of association and the extent to which these binding interactions occur.

Results and discussion

Circular dichroism

CD can gauge protein structural conformation, and is usually measured by the changes induced in the backbone amide transitions from 190–240 nm. Conformations such as α-

helix and β-sheet give distinctive CD spectra, with α-helix displaying a negative peak with maxima at 208 and 222 nm [37]. The CD spectrum of albumin (Fig. 2) is characteristic of an α-helical protein. The studies of albumin were conducted with 56MEEN only, as this compound is achiral, and as such does not complicate the CD spectra of the albumin obtained from the titration.

When 56MEEN was added to albumin (0.2 μM) in a stoichiometric quantity (1:1), the negative ellipticity at both 208 and 222 nm decreased, indicating a reduction in the α-helical nature of the albumin (Fig. 2a). No observable changes were evident in the near UV region of the CD spectrum (250–300 nm, data not shown), which can indicate changes in the tertiary structural organization of the protein [18]. In most instances in the literature, CD is only used to demonstrate a change to the secondary structure of albumin upon drug binding [26, 43]; rarely are binding constants calculated. We have highlighted these changes in the induced CD spectra (Fig. 2b) of albumin, which resulted from the subtraction of the native albumin spectrum from the measured spectrum after each incremental titration with 56MEEN. The induced changes at 192 nm are plotted as an association curve (Fig. 1 Electronic supplementary material (ESM)), with saturation reached at approximately 3×10^{-6} M (15 equivalents of metal complex). The association constant was calculated from the CD titration data using the intrinsic and Scatchard methods.

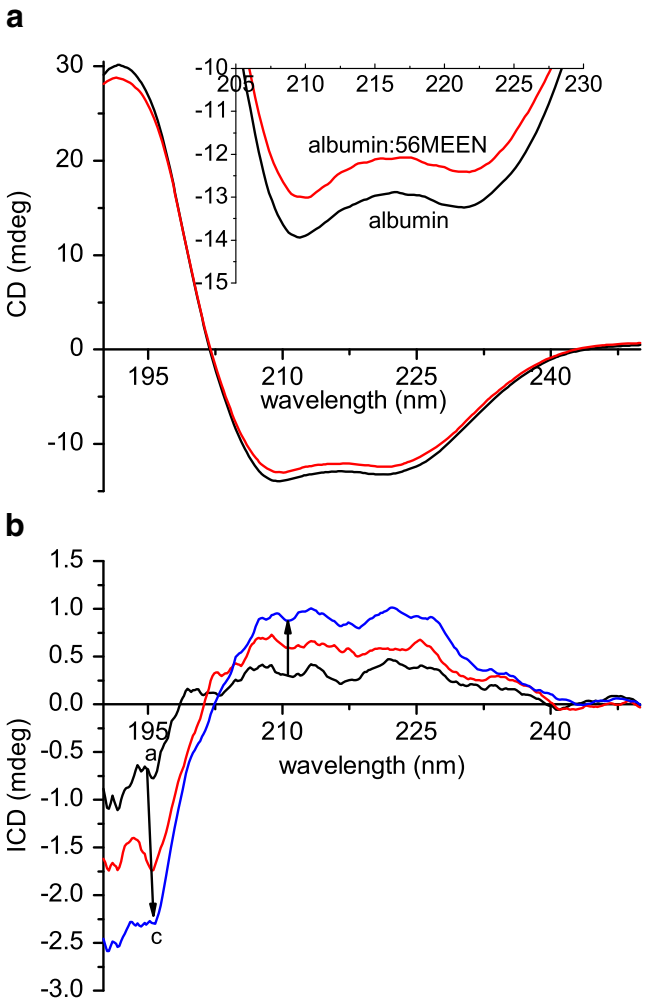

Fig. 2 **a** The CD spectrum (collected at 298 K) of albumin (2×10^{-7} M) and when combined with 56MEEN (2×10^{-7} M) the inset shows the expansion of the region 205–230 nm. **b** The induced CD spectra of albumin with (*a*) 7.7×10^{-8}, (*b*) 2.3×10^{-7} and (*c*) 5.4×10^{-7} M 56MEEN

The intrinsic method, developed by Rodger and Nordén [37] is given by:

$$\frac{[\mathrm{MC}_k]-[\mathrm{MC}_j]}{\rho_k-\rho_j}=\frac{n[P]}{\varphi}\left(\frac{\frac{[\mathrm{MC}_k]}{\rho_k}-\frac{[\mathrm{MC}_j]}{\rho_j}}{\rho_k-\rho_j}\right)+\varphi \qquad (2)$$

where [MC] is the metal complex concentration, ρ is the CD signal at a titration point, k and j are two titration points, [P] is the concentration of albumin (which remains constant), n is the number of metal complex association sites on each protein molecule and φ is a constant over the range of binding ratios. Using repeated application of Eq. 2 for each incremental titration point, the intrinsic plot of:

$$\frac{[\mathrm{MC}_k]-[\mathrm{MC}_j]}{\rho_k-\rho_j}\ \mathrm{vs}\ \frac{\frac{[\mathrm{MC}_k]}{\rho_k}-\frac{[\mathrm{MC}_j]}{\rho_j}}{\rho_k-\rho_j}$$

should be a straight line with a slope of $(n[P])/\varphi$ and y-intercept φ.

If the relationship between total, free [$\mathrm{MC_f}$] and bound [$\mathrm{MC_b}$] metal complex concentration is given by:

$$[\mathrm{MC}]=[\mathrm{MC_f}]+[\mathrm{MC_b}] \qquad (3)$$

and

$$[\mathrm{MC_b}]=\varphi\rho \qquad (4)$$

then Eqs. 3 and 4 can be used to determine [$\mathrm{MC_f}$] for each aliquot of metal complex added. These values are then applied in a Scatchard plot [38] of $r/[\mathrm{MC_f}]$ vs. r (where $r=[\mathrm{MC_b}]/[P]$), where the negative value of the gradient is the apparent binding constant (K_{app}) and the y-intercept is nK_{app}.

The intrinsic (Fig. 2 ESM) and Scatchard methods (Fig. 3 ESM) were applied to the titration data to determine the association parameters of 56MEEN with albumin (Table 1). The value of n was in agreement between both the intrinsic and Scatchard methods. The binding constant as determined from the CD titration was ~5×10^6 $\mathrm{M^{-1}}$, with approximately one 56MEEN metal complex binding site per albumin molecule. Albumin is reported to have multiple regions capable of drug binding [12] and while the CD experiments demonstrated that an interaction was indeed taking place between

Table 1 Summary of the association parameters for 56MEEN with albumin as determined from CD titration data

Intrinsic method		Scatchard method	
φ	n	n	K_{app} (M^{-1})
4.30×10^{-8}	0.91	0.93	4.7×10^{6}

56MEEN and albumin, the singular observed binding interaction can be interpreted in two ways. Either there is only one binding site capable of accommodating 56MEEN, or CD is only able to report on one binding site. As such, titration experiments using fluorescence spectroscopy were conducted with albumin and all three metal complexes (56MEEN, 56MESS, and 56MERR).

Fluorescence spectroscopy

The association parameters of 56MEEN, 56MESS, and 56MERR with albumin were also determined using fluorescence titration. The non-fluorescent platinum complex was titrated into albumin solution (1.8×10^{-6} M). The fluorescence intensity of the tryptophan residues (λ_{ex}=295 nm) was measured from 310–400 nm (λ_{max}=342 nm). The observed fluorescence intensity is quenched with increasing concentration of metal complex, with the maximum emission wavelength slightly red shifted (Fig. 3). This suggests that the interaction of the platinum complexes with albumin causes conformational changes in the protein that result in the tryptophan residue/s being brought into a more hydrophilic environment.

This quenching can be attributed to a number of processes, such as: excited state reactions, energy transfer, ground state complex formation (static quenching) and collisional processes [17]. Collisional (dynamic) quenching occurs when the fluorophore (Trp) comes into contact with the quencher during the lifetime of the excited state.Collisional quenching can be described by the Stern–Volmer equation [41]:

$$\frac{F_0}{F} = 1 + K_q\tau_0[\mathrm{MC}] = 1 + K_{SV}[\mathrm{MC}] \quad (5)$$

where F_0 is the initial fluorescence, F is the fluorescence at each titration point, K_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher (~5×10^{-9} s) [25], K_{SV} is the Stern–Volmer quenching constant. K_{SV} was determined from the linear region of a (Stern–Volmer) plot of $\frac{F_0}{F}^{-1}$ vs. [MC].

The titrations of albumin with 56MESS, 56MERR, and 56MEEN result in linear Stern–Volmer plots for metal complex concentrations less than 6×10^{-6} M^{-1} (0–3 equivalents of metal complex, Fig. 4, inset); above this ratio there is a slightly concave upwards trend observed (Fig. 4). The linearity of the Stern–Volmer plots at these lower concentrations suggests one association site is in the proximity to one of the tryptophan residues, or multiple association sites of equal accessibility. It suggests the occurrence of collisional quenching interactions between the platinum complexes and albumin. The Stern–Volmer constants for all three metal complexes were calculated for this linear region (Fig. 4, inset) and were found to be ~4×10^{4} M^{-1} (Table 2).

The quenching constants (K_q) were calculated from this linear region using Eq. 5, and were found to be ~8×10^{12} M^{-1} s^{-1} (Table 2). The maximum quenching constant resulting from collisional (dynamic) quenching of biopolymers is 2×10^{10} M^{-1} s^{-1} [48], which means that the fluorescence quenching of albumin by the metal complexes is due to the static process of association rather than collisional quenching. As such, the Stern–Volmer equation for

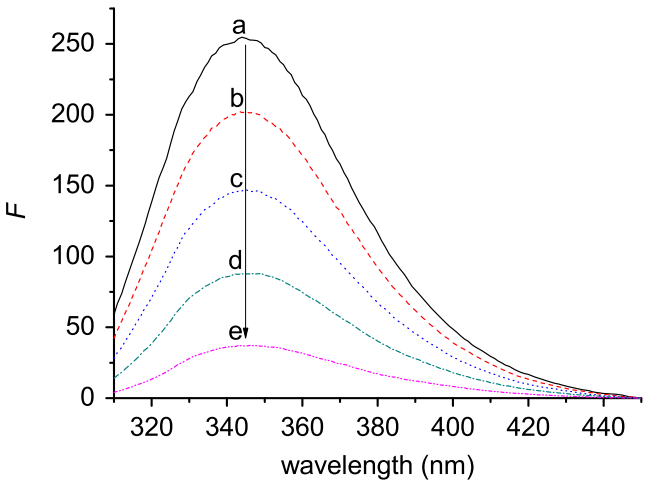

Fig. 3 The fluorescence emission spectra (λ_{ex}=295 nm) of (*a*) albumin (1.8×10^{-6} M) in the presence of: (*b*) *broken red line* 8.9×10^{-6}, (*c*) *broken blue line* 2.2×10^{-5}, (*d*) *broken green line* 4.5×10^{-5} and (*e*) *broken pink line* 8.8×10^{-5} M 56MESS

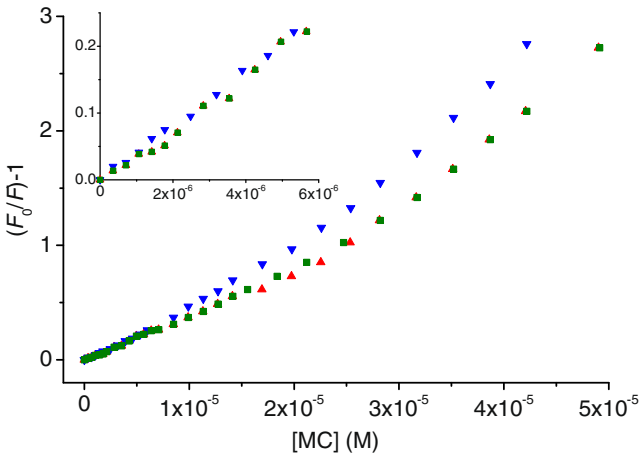

Fig. 4 The Stern–Volmer plots for the quenching of albumin (1.8×10^{-6} M) with increasing concentration of metal complexes (56MEEN *filled green square*, 56MESS *filled red upright triangle* and 56MERR *filled blue inverse triangle*). The *inset* shows the expanded region where the relationship is approximately linear

Table 2 Stern–Volmer data for the platinum complexes with albumin, as calculated from fluorescence experiments

Metal complex	K_{SV} (M^{-1})	K_q	R^2
56MESS	4.0×10^4	8.0×10^{12}	0.994
56MERR	4.2×10^4	8.4×10^{12}	0.996
56MEEN	3.8×10^4	7.6×10^{12}	0.983

collisional quenching does not apply for the interactions of the platinum complexes with albumin. As a consequence, the association constants were calculated using a double-logarithmic method (Eq. 8).

As the interactions between the metal complexes and the albumin have been shown to involve static processes (due to the large value of K_q) at low metal complex–albumin ratios, the relationship can be represented as:

$$n\mathrm{MC} + P \longrightarrow n\mathrm{MC}\cdots P \tag{6}$$

where nMC$\cdots P$ represents the quenched metal complex/albumin species. The apparent association constant (K_{app}) is given by:

$$K_{app} = \frac{[n\mathrm{MC}\cdots P]}{[\mathrm{MC}]^n[P]} \tag{7}$$

If the number of albumin molecules is equal to P_0, then $[P_0]=[n\cdots P]+[P]$ and the relationship between fluorescence intensity and albumin concentration is $[P]/[P_0]=F/F_0$, then:

$$\log_{10}\left[\frac{F_0 - F}{F}\right] = \log_{10} K_{app} + n\log_{10}[\mathrm{MC}] \tag{8}$$

the double-logarithm plot of $\log_{10}[(F_0-F)/F]$ vs. $\log_{10}$[MC] can be used to determine K_{app} and n.

The double-logarithm plots for the fluorescence titrations of albumin with 56MEEN, 56MESS, and 56MERR are linear for the whole concentration range tested (0–30 molar equivalents, Fig. 4 ESM). The binding constants were calculated to be ~2×10^5 M^{-1} (Table 3), with no apparent difference in the strength of association of 56MEEN, 56MESS, or 56MERR with albumin. These values are similar to that calculated for the association of the polyaromatic dye neutral red with albumin ($K_{app}=2.36\times10^4$ M^{-1}) [39]. There are very few reports in the literature of studies of platinum(II) complexes with BSA; however, structurally similar nickel(II) compounds have been reported to have binding constants in the order of 10^4–10^5 M^{-1}, as determined by fluorescence spectroscopy [6]. The number of binding sites on albumin for each of the metal complexes is ~1.1.

Table 3 The apparent association constant (K_{app}) and the number of binding sites (n) for each metal complex with albumin, as calculated from double-logarithm plots

Metal complex	K_{app} (M^{-1})	n	R^2
56MEEN	2.1×10^5	1.14	0.996
56MESS	1.1×10^5	1.08	0.990
56MERR	2.4×10^5	1.14	0.997

While both CD (of 56MEEN) and fluorescence titrations (of 56MEEN, 56MESS, and 56MERR) demonstrated that the addition of the metal complex caused changes to the secondary structure of the protein, each suggested only one metal complex binding site with differing apparent association constants measured by each technique. We postulate that the two techniques are measuring either two different and unique association events or fluorescence is measuring an average of two different binding interactions where one is the same as measured by CD. The possibility of multiple associations was further investigated using pulsed gradient spin-echo NMR.

NMR diffusion measurements

The concentration of 56MEEN, 56MESS, and 56MERR were varied from 2–30 mM, and the diffusion coefficients (D_{obs}) of the metal complexes measured using PGSE NMR in the presence of 0.5 mM albumin in unbuffered D_2O at 298 K. Lower metal complex concentrations could not be measured due to insufficient signal-to-noise. The attenuation of the metal complex signal was single exponential in all cases (Fig. 5, ESM), indicating that the metal complex binds to the albumin with fast exchange kinetics (on the timescale of the

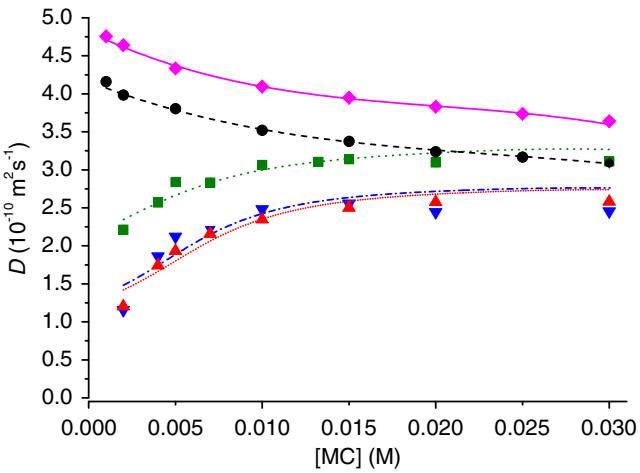

Fig. 5 The observed diffusion coefficient as concentration of free 56MEEN (*filled pink diamond*, *solid pink line*), free 56MESS/56MERR (*filled black circle*, *broken black line*), and 56MEEN (*filled green square*, *dotted green line*), 56MESS (*filled red upright triangle*, *dotted red line*) and 56MERR (*filled blue inverse triangle*, *blue dash–dot line*) when combined with 0.5 mM albumin. For the free metal complexes, the curve is the result of third-order polynomial regression onto the data (Eqs. 13 and 14). For the samples containing albumin, the curves are the result of regressing the two-site model (Eq. 10) onto the data. The poor fit to the data indicates that the model is too simplistic and/or the assumptions of the model are incorrect

NMR diffusion measurement, Δ). At lower metal complex concentrations, D_{obs} was close to the diffusion coefficient of free albumin (i.e., D_b, 0.64×10^{-10} m^2s^{-1}) [36] indicating that the majority of the metal complex was bound to albumin. At higher metal complex concentrations (~15 mM), the diffusion coefficient of the metal complex is ~2.5×10^{-10} m^2s^{-1} (Fig. 5, Table 1 ESM). At these higher concentrations, the majority of the added metal complex is free in solution.

The two-site model can be used to determine association parameters, where it is assumed that a small molecule, (which in this case is metal complex) can bind to any of the n identical independent association sites on a protein [27]:

$$P + \mathrm{MC} \underset{}{\overset{K_{app}}{\rightleftharpoons}} P\cdots\mathrm{MC} \tag{9}$$

The relationship between the population weighted diffusion coefficient of the metal complex (D_{obs}), the mole fraction of bound metal complex (P_b), the diffusion coefficients of the free (D_f) and bound metal complex (D_b, which in this case is taken as the albumin diffusion coefficient) and the concentrations of the metal complex and the protein is given by:

$$D_{obs} = (1 - P_b)D_f + P_bD_b \tag{10}$$

where

$$P_b = \alpha - \sqrt{\alpha^2 - \beta} \tag{11}$$

and

$$\alpha = \frac{[\mathrm{MC}]+n[P]+\frac{1}{K_{app}}}{2[\mathrm{MC}]} \text{ and } \beta = \frac{n[P]}{[\mathrm{MC}]}. \tag{12}$$

Previous studies have shown that D_f of 56MESS/56MERR decreases with increasing metal complex concentration due to the self-aggregation of the metal complex in solution [24]. The diffusion data of the free metal complexes (Fig. 5) were interpolated with a third-order polynomial to provide D_f (m^2s^{-1}) at all MC (M) for each metal complex, viz.

$$\begin{aligned} D_{f\,56MEEN} = {} & 4.82\times10^{-10} - 1.11\times10^{-8}(\mathrm{MC}) \\ & + 4.60\times10^{-7}(\mathrm{MC})^2 - 7.55 \\ & \times10^{-6}(\mathrm{MC})^3 \end{aligned} \tag{13}$$

$$\begin{aligned} D_{f\,56MESS/RR} = {} & 4.16\times10^{-10} - 9.00\times10^{-9}(\mathrm{MC}) \\ & + 3.13\times10^{-7}(\mathrm{MC})^2 - 4.44 \\ & \times10^{-6}(\mathrm{MC})^3 \end{aligned} \tag{14}$$

Non-linear regression of Eq. 10 on to the data was used to estimate the values of K_{app} and n for each metal complex with albumin.

The analysis of the diffusion data using the two-site model (Eqs. 10, 11, and 12); resulted in a relatively poor fit (Fig. 5, Table 4). This indicates that the association is in reality much more complex than that described by this simplistic model. The analysis estimated the number of association sites to be ~10±2 with an average association constant in the order of ~10^2–10^3 M^{-1} (Table 4); however, the error calculated for K_{app} was of the same order of magnitude. No difference was observed in the association parameters of the three metal complexes, within experimental error. In comparison to the association of other small molecules examined by this technique, the metal complexes bind relatively strongly to albumin. Although PGSE has been used as a method to identify and measure binding constants [29, 47], it has not been applied to this class of compounds before. In comparison to salicylate, the metal complexes bind relatively strongly to albumin. The association constant of salicylate to albumin is only 33 M^{-1}, with 33±3 equivalent association sites [36], and the binding constant of ibuprofen 59 M^{-1} with ~50 binding sites [28].

There are a number of possible explanations for the relatively poor fit of the data using the two-site model. The assumption of multiple equivalent association sites on the protein is likely to be unrealistic; also, at the concentration range measured (in millimolars) metal complex aggregates may be the dominant form present in solution [24]. These aggregates are not accounted for in the two-site model and may have different interactions with the protein compared to single free metal complex ions (which may be dominant at a lower concentration range, e.g., micromolars).

Comparison of association constant determinations

Three different spectroscopic methods were used in the examination of the interactions of the three platinum complexes with albumin (Table 5). The comparison of CD, fluorescence spectroscopy and PGSE NMR is of interest as these three spectroscopic techniques are often used in isolation for analysis of drug–protein interactions, rather than as complementary techniques.

CD was used to monitor the reduction in the α-helical content of the protein upon titration with 56MEEN. While

Table 4 Association parameters of 56MEEN, 56MESS and 56MERR with albumin, as measured using PGSE diffusion NMR and calculated using the two-site binding approximation

Metal complex	K_{app} (M^{-1})	n	R^2
56MEEN	4×10^2	9.99±1.24	0.852
56MESS	9×10^2	10.84±1.21	0.911
56MERR	9×10^2	10.17±1.99	0.727

Errors are given in parentheses

Table 5 Summary of the average binding parameters calculated for 56MEEN with albumin using various spectroscopic techniques

Method	56MEEN–albumin K_{app} (M^{-1})	Approximate number of binding sites
Circular dichroism	5×10^6	1
Fluorescence	2×10^5	1
PGSE	4×10^2	10

an association constant of 10^6 M^{-1} was estimated for the calculated association site, there are non-helical regions of the protein in which binding events cannot be monitored using this method. In addition, the chiral complexes (56MESS and 56MERR) were unable to be studied due to the complexity that this adds to the CD spectrum. As such, the fluorescence titration method was used for 56MEEN, 56MESS, and 56MERR, as it is the method predominantly used in the literature for the determination of association constants to albumin [2, 39, 43]. For these three platinum complexes, this method gave calculated association constants of ~2×10^5 M^{-1} and ~1.1 association sites. The association constants calculated from fluorescence spectroscopy are of the same order of magnitude as a variety of other exogenous compounds [31].

Both CD and fluorescence spectroscopy indicated one metal complex binding site per albumin molecule; however, each technique is only able to measure binding events occurring in specific (and often different) regions. It is likely that the metal complex associations measured between the two techniques were indeed different, with the association constants calculated differing by an order of magnitude. It is possible that there are additional metal complex associations that are occurring in regions that are unable to be measured by either of these techniques.

PGSE NMR is a very useful technique for the measurement of association constants, as it measures the population weighted diffusion coefficient of the metal complex, which can either in be the free state, or bound to the protein. This measurement is an average over all binding sites. The two-site model was shown to be an overly simplistic representation of the interactions between the metal complexes and the albumin. This model assumes that all association sites are equivalent, which is unlikely, and as such, the calculated association constant for the method will be representative of an average of the different associations occurring, of which only a selected number are able to be monitored by fluorescence and CD. Due to the numerous sub-domains of albumin, it is likely that there are multiple metal complex association sites on the albumin, each with a unique association constant. These multiple metal complex binding sites may be of great importance in the pharmacokinetics of this group of compounds, and may be beneficial for describing toxicity and drug distribution profiles from in vivo studies.

Experimental

Materials

56MEEN, 56MESS, and 56MERR were synthesized as previously described [49]. BSA was purchased from Sigma-Aldrich (A7906, 98 % lyophilized powder) and was used without further purification. Wilmad NMR tubes were used for all PGSE experiments. The concentration of albumin was determined spectrophotometrically using the molar extinction coefficient of 4.4×10^4 M^{-1} cm^{-1} at 279 nm [13].

Circular dichroism

Circular dichroism measurements were recorded on a Jasco J-810 CD spectropolarimeter operating at room temperature (~298 K) using a cell length of 1 cm. Titrations were performed by titration of 56MEEN solution (1–2 μL, 100–200 μM) into a solution of albumin (~0.2 μM, 2.6 mL) up until a final metal complex concentration of ~14 μM. The CD spectrum was recorded from 190–250 nm (five accumulations) after each titration.

Fluorescence spectroscopy

Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrometer operating at room temperature (~298 K), using a scan rate of 120 nm min^{-1}, an averaging time of 0.5 s, data collection every 1 nm, and with excitation and emission slit lengths set to 5 nm. A solution of albumin (~2 μM, 3 mL) was then prepared in H_2O. Metal complex solution (56MEEN, 56MESS, and 56MERR, 0.25–2.5 μL, ~4.25 mM) was titrated into the albumin solution, up until a final metal complex concentration of ~60 μM. After each titration the fluorescence intensity was measured (λ_{ex}=295 nm) from 310–400 nm, with the fluorimeter zeroed on H_2O at 310 nm before each measurement. The concentrations were corrected at each titration point for the dilution factor; however, over the course of the whole experiment this was only 1 %.

PGSE NMR spectroscopy

^1H NMR spectra were recorded on a Bruker Avance 400 spectrometer, operating at a ^1H resonance frequency of 400.13 MHz. Spectra were obtained with a 5-mm BBO probe using a spectral width of 2,400 Hz, an acquisition time of 1.0 s, a 90° pulse width of 14.3 μs and a recycle delay of 5 s. A line broadening of 0.5 Hz was applied prior to Fourier transformation. Measurements were performed at 298 K. Spectra were internally referenced to the residual HDO peak at 4.77 ppm. Diffusion measurements were performed using a modified Hahn spin–echo-based PGSE pulse

sequence, optimized to reduce signal loss due to spin–spin relaxation [33, 40]. The separation between the leading edges of the gradient pulses (Δ), which defines the time scale of the diffusion measurement, was set to 20 ms. The gradient pulse width, δ, was set to ~6.5–8.0 ms. Echo attenuation of the metal complex resonance/s (either aromatic or methyl) was measured at 16 different gradient amplitudes (g, between 0–0.40 T m^{-1}) in each diffusion measurement. For a single species undergoing free isotropic diffusion the attenuation (E) of the spin–echo signal is given by [34, 40]:

$$E = e^{-\gamma^2 g^2 \delta^2 D\left(\Delta - \frac{\delta}{3}\right)} \quad (14)$$

where γ is the gyromagnetic ratio of the observed nucleus. The diffusion coefficient and the associated error were determined using a non-linear least squares fit of Eq. 14 onto the PGSE data using OriginPro 8 (OriginLab, MA).

Conclusions

Up until now, there have been limited studies of platinum anticancer complexes with albumin, as the primary cellular target is believed to be DNA and so these interactions are more prominently reported in the literature. The platinum metallointercalators investigated here all bind to albumin, causing changes to the secondary structure of the protein, as demonstrated by circular dichroism experiments with 56MEEN and fluorescence spectroscopy with all three metal complexes. There does not appear to be any significant differences in the interactions with albumin between the three metal complexes, as measured by fluorescence and PGSE. The association constants have been determined by a number of spectroscopic methods. CD titrations conducted with 56MEEN estimated a 0.93 association sites and an association constant in the order of 10^6 M^{-1}. Fluorescence spectroscopy measured a similar number of association sites (~1); however, the association constants determined were lower (~10^5 M^{-1}). PGSE diffusion NMR is sensitive to all exchange processes; however, the two-site model is not an adequate representation of this complex system. As such, the binding constant of ~10^2–10^3 M^{-1} can only be determined as an average of approximately ten different associations that are occurring.

It is unlikely that the protein has only a single class of binding site. Consequently, at higher metal complex concentrations, the interactions of the metal complex with the protein is likely to be fundamentally different than at lower concentrations since stronger binding sites will already be saturated. Further, at higher concentrations, the metal complex can self-aggregate leading to an essentially new molecule with different binding properties. Thus, it is reasonable that the PGSE experiments, which are conducted at much higher metal complex concentrations (in millimolars) compared to CD and fluorescence experiments (in micromolars), give rise to significantly different binding data. Importantly, the higher concentrations are more relevant for understanding drug administration and albumin formulation, whereas the lower concentrations are more physiologically relevant.

The multiple binding sites measured on albumin may have an important role in the in vivo behavior of these platinum complexes, with the weaker binding sites allowing the drug molecules to disassociate from the albumin, whereas the stronger binding sites may only release the drug as the protein is degraded/metabolized.

Future work will explore techniques such as isothermal titration calorimetry and synchronous fluorescence to further probe the drug interactions with albumin. We will also focus on developing more complex models to explain the results obtained from the PGSE experiments. Further experiments could utilize techniques such as multidimensional NMR in order to gain further information about the location of metal complex association sites on albumin.

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